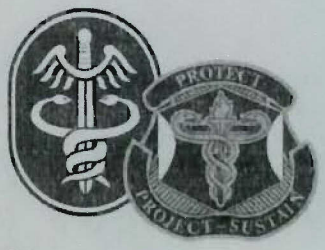

U.S. Army Medical Research Institute of Chemical Defense

USAMRICD-TR-05-03

Development of a Protocol for Triple Labeling of MAP-2, Fluoro-Jade, and DAPI in a Single Paraffin-embedded Rat Brain Section

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April 2005

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
April 2005	Technical Report	January 2004 - June 2004

4. TITLE AND SUBTITLE
Development of a Protocol for Triple Labeling of MAP-2, Fluoro-Jade, and DAPI in a Single Paraffin-embedded Rat Brain Section

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
61384

6. AUTHOR(S)
Tompkins, CP, Fath, DM, Hamilton, TA, Petrali, JP, and Kan, RK

5d. PROJECT NUMBER
TC1

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-UV-CC
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER
USAMRICD-TR-05-03

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Institute of Chemical Defense
ATTN: MCMR-UV-RC
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The present study was conducted to develop a protocol for triple labeling of MAP-2, Fluoro-Jade, and DAPI to evaluate the pathological consequences of nerve agent toxicity in the rat model. Brains of rats subcutaneously challenged with a single injection of soman were formalin-fixed, paraffin-embedded, and sectioned. Following microwave antigen retrieval, sections were stained with MAP-2 monoclonal antibody to evaluate changes in dendritic processes, with Fluoro-Jade to examine the degree of neuronal cell degeneration or death, and with DAPI to assess the number of viable nuclei. Control hippocampus showed typical MAP-2 immunofluorescence localized predominantly in dendritic processes, numerous DAPI positive nuclei, and absence of Fluoro-Jade positive neurons. In the hippocampus of soman-exposed rats, Fluoro-Jade positive cells were detected in areas where MAP-2 immunoflourescence was lost and the number of DAPI positive nuclei was decreased. The results suggest that our triple labeling technique is effective for simultaneously examining MAP-2, Fluoro-Jade, and DAPI fluorescence in the same section, thus reducing the number of sections necessary to comprehensively evaluate dendritic pathology, neurodegeneration, and neuronal loss. More importantly, the technique permits precise anatomical merging of MAP-2, Fluoro-Jade, and DAPI images, which cannot be accomplished when these stains are performed separately on serial sections.

15. SUBJECT TERMS
Triple labeling, immunofluorescence, MAP-2, Fluoro-Jade, and DAPI

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Robert K. Kan
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	16	19b. TELEPHONE NUMBER *(include area code)* 410-436-2334

ABSTRACT

The present study was conducted to develop a protocol for triple labeling of MAP-2, Fluoro-Jade, and DAPI to evaluate, using immunofluorescence and histofluorescence, the pathological consequences of nerve agent toxicity in the rat model. Male Sprague Dawley rats were subcutaneously challenged with a single injection of 1.6 LD_{50} of soman. At 24 hr post-injection, rats were deeply anesthetized with sodium pentobarbital and then transcardially perfused with normal saline, followed by 10% formalin. Brains, harvested immediately after perfusion, were post-fixed in formalin, cut coronally into 3 mm slabs, processed in paraffin, and sectioned at 4 μm. Following microwave antigen retrieval, sections were stained with MAP-2 monoclonal antibody to evaluate changes in dendritic processes, with Fluoro-Jade to examine the degree of neuronal cell degeneration or death, and with DAPI to assess the number of viable nuclei. Control hippocampus showed typical MAP-2 immunofluorescence localized predominantly in dendritic processes, numerous DAPI positive nuclei, and absence of Fluoro-Jade positive neurons. In the hippocampus of soman-exposed rats, Fluoro-Jade positive cells were detected in areas where MAP-2 immunoflourescence was lost and the number of DAPI positive nuclei was decreased. The results suggest that our triple labeling technique is effective for simultaneously examining MAP-2, Fluoro-Jade, and DAPI fluorescence in the same section, thus reducing the number of sections necessary to perform a comprehensive evaluation of dendritic pathology, neurodegeneration, and neuronal loss. More importantly, the technique permits precise anatomical merging of MAP-2, Fluoro-Jade, and DAPI images, which cannot be accomplished when these stains are performed separately on serial sections.

ACKNOWLEDGEMENTS

The authors acknowledge branch chief MAJ Scot Estep for his support. The first author also acknowledges the U.S. Army Medical Research Institute of Chemical Defense for providing additional support of this research through Battelle.

CONTENTS

1. INTRODUCTION

Microtubule-associated protein 2 (MAP-2) immunohistochemistry and Fluoro-Jade histofluorescence are commonly used as investigative and diagnostic markers of neuronal injury. MAP-2 is the most abundant neuron-specific cytoskeletal protein in the brain, localized mostly in the dendritic processes (Caceres *et al.*, 1984; De Camilli *et al.*, 1984). Loss of MAP-2 immunoreactivity has been shown to be a sensitive marker of brain damage following lateral fluid percussion (Hicks *et al.*, 1995), cerebral ischemia (Kitagawa *et al.*, 1989), and chemical warfare nerve agent intoxication (Ballough *et al.*, 1995). Fluoro-Jade is an anionic fluoroscein derivative that stains the cell bodies, dendrites, axons, and axon terminals of degenerating neurons but does not stain healthy neurons (Schmued and Hopkins, 2000).

One advantage of fluorescent markers is their sensitivity, which in part is due to the fact that bright fluorochromes are viewed in relation to a dark background (Schmued et al., 1982). The dark field is a disadvantage, however, when it is necessary to determine the precise location of fluorescent cells in tissue sections. Therefore, a fluorescent counterstain, such as 4,6-diamidino-2-phenylindole hydrochloride (DAPI), can be used. DAPI is a polycationic molecule with a high affinity for nuclear DNA, which permits visualization of nuclei in histochemical preparations (Sanna *et al.*, 1992). Because of its compatibility with the most commonly used fluorochromes, such as fluorescein, DAPI is ideal for use as a fluorescent counterstain (Sanna *et al.*, 1992; Hoff, 1988; Tarnowski *et al.*, 1991). In addition, short incubation times are sufficient to provide an intense signal with virtually no background (Sanna *et al.*, 1992). As a result of these properties, double and triple labeling can be performed.

Our laboratory is interested in developing a working protocol for labeling of MAP-2, Fluoro-Jade, and DAPI in paraffin-embedded rat brain sections. To our knowledge, no reports exist on triple labeling of MAP-2, Fluoro-Jade, and DAPI in routinely formalin-fixed, paraffin-embedded rat brain sections. Once established, the protocol would permit simultaneous evaluation of dendritic pathology (MAP-2), neurodegeneration (Fluoro-Jade), and neuronal loss (DAPI), in the same tissue section, following soman-induced seizure-related brain injury. For this study, we chose to examine the CA1 cell field of the hippocampus, an area known to sustain considerable damage following acute soman exposure (Carpentier *et al.*, 1991).

2. MATERIALS AND METHODS

2.1 Animals

Twelve (6 control and 6 experimental) male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 250-350g, were used in the study.

2.2 Treatment

Animals were pretreated with the oxime HI-6 (125mg/kg, i.p.) 30 min prior to soman challenge (180μg/kg, s.c. = 1.6 LD_{50}). One minute after soman injection, animals

were treated with atropine methyl nitrate (2.0 mg/kg, i.m.). The dose of soman used was previously reported to induce seizure in 100% of the animals (Shih *et al.*, 1991). HI-6 and atropine were used to increase the survival of animals. Control animals received an equivalent volume of vehicle, HI-6, and atropine. At 24 hr after soman exposure, animals were deeply anesthetized with sodium pentobarbital (>75 mg/kg, i.p.) and then transcardially perfused with 0.9% saline, followed by 10% neutral phosphate buffered formalin.

2.3 Tissue Preparation and Processing

Brains were removed and post fixed in 10% neutral phosphate buffered formalin for 18 hr at 4°C. Brains were then cut coronally into 3-mm slabs using a rat brain matrix (ASI Instruments, Warren, MI), processed in paraffin, sectioned serially at 5μm, and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). After drying at room temperature for 24 hr, sections were stained sequentially with MAP-2, Fluoro-Jade, and DAPI. This order of staining was determined to be optimal.

2.4 Microwave Pretreatment

Sections were dewaxed in xylene, hydrated to distilled water, and then incubated in 5% hydrogen peroxide for 15 min at room temperature to suppress endogenous peroxidase activity. Following thorough washing in running tap water (5 min), sections were rinsed in distilled water. Ten mM citric acid monohydrate (Sigma-Aldrich; St Louis, MO; Lot 30H-0627) was used as an antigen retrieval solution. Citric acid solution was prepared according to formula (.21g/100ml) and adjusted to pH 6.0 by adding 2N NaOH, while monitoring with a pH meter (Beckman Instruments, Fullerton, CA). MAR procedure was performed as described in USAMRICD-TR-02-06 (Pleva *et al.*, 2002; Kan *et al.*, 2005). Sections were boiled in a microwave oven (Pelco 3440 Max, Ted Pella, Inc., Redding, CA) in plastic Coplin jars for two 5-min cycles, with the power of the microwave set at 100%. Each cycle was broken into two equal time periods of 2.5 min so that more AR solution could be added to compensate for loss due to boiling over and to avoid drying out the tissue sections. Following two cycles of boiling in the microwave for a total time of 10 min, sections were allowed to cool at room temperature for a minimum of 20 min prior to processing for MAP-2 immunohistochemistry.

2.5 MAP-2 Immunohistochemistry

Following microwave antigen retrieval, sections were rinsed twice with distilled water and then oxidized in 0.06% potassium permanganate for 10 min. After rinsing thoroughly in distilled water, brain sections were rinsed in phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO; Lot 12K8203), and incubated in 5% normal horse serum derived from the host for the secondary antibody for 30 min at 4°C to block non-specific binding sites of tissue immunoglobulins to secondary antibody. Sections were then incubated in mouse MAP-2 monoclonal antibody (Clone AP18, 1:100; NeoMarkers, Fremont, CA) for 18 hr at 4°C. Following two washes with PBS, sections were incubated with biotinylated secondary antibody (1:200 dilution) (Vector,

Burlingame, CA) for 1 hr at room temperature, washed twice with PBS, and allowed to react with streptavidin conjugated with Alexa-Fluor® 594 (1:200 dilution) (Molecular Probes, Eugene, OR) for 30 min at room temperature. Negative control sections were treated in an identical manner except that incubation in primary antibody or microwave pretreatment was omitted.

2.6 Modified Fluoro-Jade Staining for Use with Immunohistochemistry

This method was adapted from that originally described by Schmued *et al.* (Schmued *et al.*, 1997). Following incubation in Alexa Fluor 594, sections were rinsed in PBS, distilled water, and then stained in 0.001% Fluoro-Jade solution (Histo-Chem, Jefferson, AR) for 30 min in the dark. After staining, sections were rinsed 3 times for 1 min each in distilled water. Excess water was drained off, and the slides were air-dried on a staining tray overnight at room temperature.

2.7 Fluorescent Counterstaining

After drying the slides, one drop of VECTASHIELD® mounting medium, containing 4,6-diamidino-2-phenylindole hydrochloride (DAPI), was dispensed onto each section. The sections were then coverslipped, allowing the mounting media to disperse over the entire section. Following 15 min incubation in DAPI, the slides were decoverslipped in distilled water, air-dried overnight, and coverslipped with Permount® (Fisher Scientific, Fair Lawn, NJ). Slides were evaluated using an Olympus BX61 fluorescent microscope (Olympus, Melville, NY) equipped with an Olympus DP70 digital camera. Images were then merged using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). All photomicrographs were taken with a 40X objective.

3. RESULTS

Results are illustrated in Figure 1 and Figure 2. In the CA1 region of the hippocampus, control sections showed intense red MAP-2 immunofluorescence (Figure 1A), only occasional Fluoro-Jade positive neurons (Figure 1C), and numerous brilliant blue DAPI-labeled nuclei (Figure 1E). When the three images were overlayed (Figure 2A), typical MAP-2 immunoreactivity was seen predominantly in dendritic processes. DAPI permitted easy identification of nuclei and facilitated assessment of gross cell morphology. Following soman intoxication, loss of MAP-2 immunoreactivity was observed in all strata (Figure 1B). In the stratum radiatum, apical dendrites of pyramidal neurons were fragmented and convoluted. Within the stratum pyramidal, degenerating neurons were labeled strongly with Fluoro-Jade (Figure 1D), and loss of DAPI positive nuclei was observed (Figure 1F). In addition, nuclei appeared shrunken and condensed compared to nuclei in control sections. After merging the images (Figure 2B), the presence of Fluoro-Jade positive cells was noted to coincide with absence of MAP-2. Fluoro-Jade labeling appeared to be specific to degenerating neurons. In general, background staining was faint, while degenerating neurons and their processes stained conspicuously.

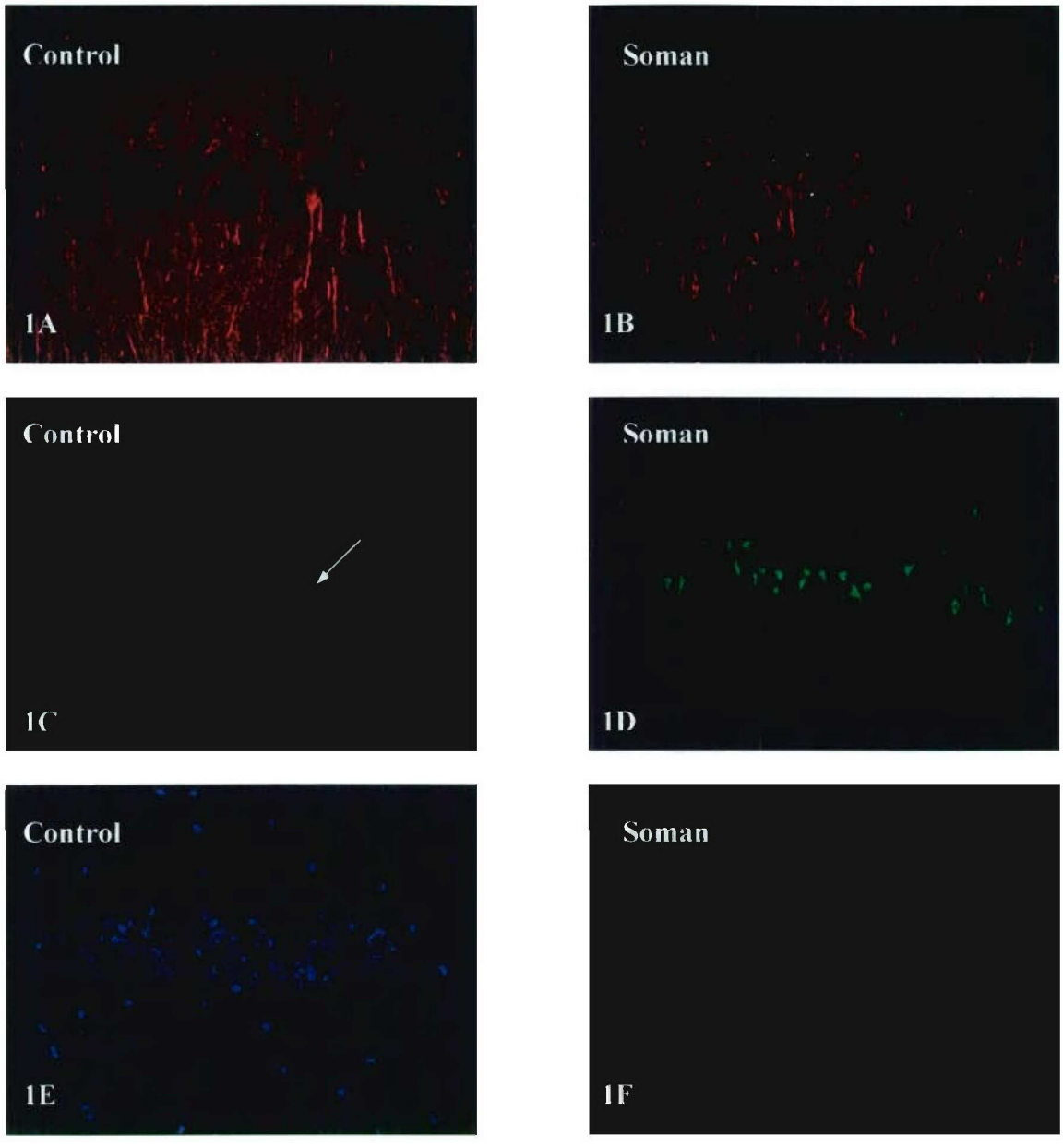

Figure 1. MAP-2 (1A, 1B), Fluoro-Jade (1C, 1D), and DAPI (1E, 1F) staining in the CA1 region of the hippocampus in control and soman-exposed brain. Control sections show normal patterns of MAP-2 staining (1A), only occasional Fluoro-Jade positive neurons (arrow, 1C), and numerous DAPI positive nuclei (1E). Following soman exposure, sections exhibit loss of MAP-2 (1B), a marked increase in Fluoro-Jade positive neurons (1D), and a decrease in the number of DAPI positive nuclei (1F).

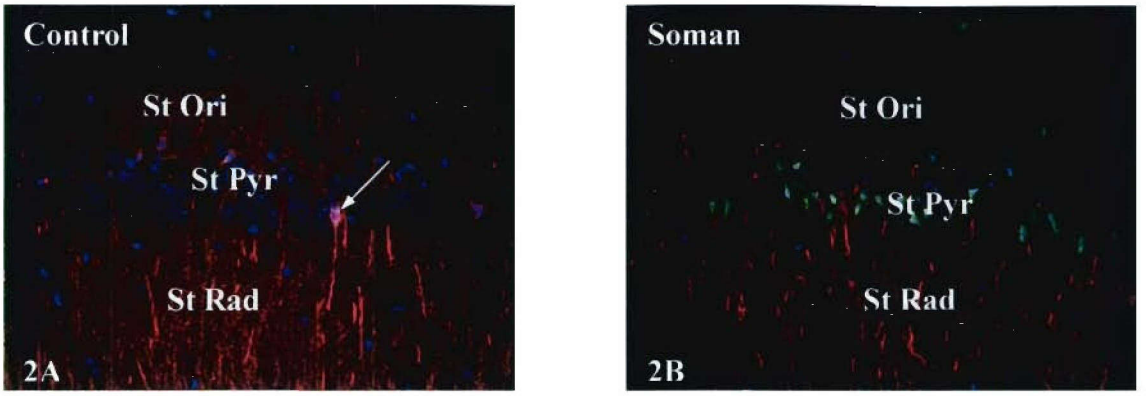

Figure 2. Combined MAP-2, Fluoro-Jade, and DAPI images (2A, 2B). Control hippocampus shows MAP-2 staining located primarily in dendritic processes and only occasional Fluoro-Jade positive neurons in stratum pyramidal (arrow, 2A). Loss of MAP-2 was observed in all three layers of the hippocampal CA1 subregion following soman exposure (2B). In the stratum radiatum, apical dendrites were fragmented. Within the stratum pyramidal, degenerating neurons were intensely labeled with Fluoro-Jade, and loss of DAPI positive nuclei was observed. St Rad, Stratum Radiatum; St Pyr, Stratum Pyramidal; St Ori, Stratum Orien.

4. CONCLUSIONS

Results demonstrate that the established staining procedure is effective for triple labeling of MAP-2, Fluoro-Jade, and DAPI in the same tissue section. Triple labeling with MAP-2, Fluoro-Jade, and DAPI can be used successfully as an approach to examining soman-induced seizure-related pathology in the rat model. Importantly, this technique permits precise anatomical merging of MAP-2, Fluoro Jade, and DAPI digital images so that dendritic pathology, neurodegeneration, and neuronal loss can be evaluated simultaneously in the same tissue section. In addition, this technique reduces the number of sections required to perform such a comprehensive analysis. Concurrent visualization of MAP-2, Fluoro-Jade, and DAPI will undoubtedly aid in studies aimed to assess the progression of neuropathology following exposure to soman or resulting from brain injury induced by trauma or ischemia.

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APPENDIX

Triple Labeling with MAP-2, Fluoro-Jade, and DAPI

1. Deparaffin sections and hydrate to dH_2O.
    a. Xylene (3 changes, 30 min each)
    b. Absolute EtOH (2 changes, 15 min each)
    c. 95% EtOH (2 changes, 5 min each)
    d. 70% EtOH (5 min)
    e. dH_2O (5 min)

2. Quench sections with 5% H_2O_2 for 15 min to eliminate endogenous peroxidase activity.

    200 mlof 5% H_2O_2 solution: 167ml PBS
    33ml 30% H_2O_2

3. Rinse in running tap H_2O, 5 min.
4. Rinse in dH_2O, 5 min.
5. Microwave pretreatment to expose antigens:

    Boil sections in 10mM citric acid, pH 6.0 (2.1 g citric acid monohydrate in 1 liter dH_2O, adjust to pH 6.0 with 2M NaOH), in microwave for 5 min. Treat 2X.

    Cool for 20 min after last treatment.

6. Rinse in dH_2O, 5 min.
7. 0.06% potassium permanganate ($KMnO_4$), 10 min.

    200 ml of 0.06% potassium permanganate: 200ml dH_2O
    120mg $KMnO_4$

8. 2 rinses with dH_2O, 1 min each.
9. Rinse in PBS, 5 min.
10. Incubate sections in blocking serum (5% normal serum from animals that made the secondary antibody) for 30 min at 4°C (Vector, Burlingame, CA).

    10 ml solution: 9500µl PBS
    500µl (5%) normal serum

11. Incubate sections in primary antibody (1:100 dilution) for 18 hours at 4°C.

    10 ml solution: 9900µl PBS

100µl primary antibody [MAP-2 AP-18 (NeoMarkers, Fremont, CA)]

12. 2 rinses with PBS, 5 min each.
13. Incubate sections in biotinylated secondary antibody (1:200 dilution) solution at 37°C for 1 hour (Vector, Burlingame, CA).

    10 ml solution: 9950µl PBS
    50µl biotinylated antibody

14. 2 rinses with PBS, 5 min each.

***** **From this step on, perform as much of the protocol as possible away from light.**

15. Incubate sections in fluorescent streptavidin conjugate (1:200 dilution) solution at 37°C for 30 min (Molecular Probes, Eugene, OR).

    1 ml solution: 995µl PBS
    5µl Alexa-Fluor® 594

16. Rinse in PBS (5 min)
17. Rinse in dH_2O (5 min)
18. Incubate sections in 0.001% Fluoro-Jade solution for 30 min in the dark (Histo-Chem, Jefferson, AR).

    FJ stock solution (good for 2 months at 4°C)

Fuoro-Jade	50mg
Autoclaved H_2O	500ml

    FJ working solution

FJ stock solution (0.01%)	20ml
0.1% acetic acid	180ml (180µl acetic acid in 180ml dH_2O)

    Prepare solution in dim light.

19. 3 rinses in dH_2O, 1 min each.
20. Flat dry at room temperature overnight.
21. Coverslip using VECTASHIELD® mounting medium with DAPI, 15 min (Vector, Burlingame, CA)
22. Decoverslip in dH_2O
23. Flat dry at room temperature.
24. Mount slides with Permount® (Fisher Scientific, Fair Lawn, NJ).